

Effects of MK-801 (dizocilpine) on Brain Cell Membrane Function and Energy Metabolism in Experimental *Escherichia coli* Meningitis in the Newborn Piglet

We evaluated the efficacy of non-competitive N-methyl-D-aspartate receptor antagonist MK-801 (dizocilpine) as an adjuvant therapy in experimental neonatal bacterial meningitis. Meningitis was induced by injecting 10^6 colony forming units of *Escherichia coli* into the cisterna magna. MK-801 3 mg/kg was given as a bolus intravenous injection, 30 min before the induction of meningitis. MK-801 did not down-modulate the inflammatory parameters, such as increased intracranial pressure, cerebrospinal fluid (CSF) leukocytosis, increased lactate and TNF- α levels in the CSF, and hypoglycorrhachia observed in the meningitis group. MK-801 did not significantly attenuate the elevated glutamate concentration in the CSF. However, MK-801 showed some neuroprotective effects as evidenced by significant attenuation of cerebral lipid peroxidation products (conjugated dienes) and increase of brain high-energy phosphate compounds (ATP and PCr). Improvement in cerebral cortical cell membrane Na⁺, K⁺-ATPase activity did not reach a statistical significance. These results suggest that MK-801 was effective in ameliorating brain injury in neonatal bacterial meningitis, although it failed to attenuate the inflammatory responses.

Key Words : Meningitis, Bacterial; Excitatory Amino Acid Antagonists; Energy Metabolism; Brain; Animals, Newborn

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INTRODUCTION

Bacterial meningitis remains as an important clinical problem at any age, and is accompanied by significantly high mortality and morbidity despite the development of highly active new antibiotics and intensive care medicine. The prognosis is particularly poor in neonates with mortality rates of 20-40% and long-term neurologic sequelae in up to 50% of survivors (1-3). Therefore, better understanding of the pathophysiology of neonatal bacterial meningitis would be necessary to develop new adjuvant therapies that can improve the outcome of this disease.

Excitatory amino acids (EAA) are increasingly implicated in the pathogenesis of neuronal injury induced by a variety of central nervous system insults, such as ischemia, trauma, hypoglycemia and epilepsy (4-7). Glutamate is the most abundant excitatory amino acid neurotransmitter in human, being stored in presynaptic vesicles and released in response to presynaptic neuronal membrane depolarization, and the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor is considered to play a pivotal role in enabling glutamate to express its neurotoxic potential in a variety of neurologi-

cal disorders. Binding of glutamate to specific NMDA receptors opens transmembrane ion channels, with a subsequent excessive increase in intracellular sodium, calcium, and water, which elicits an initial swelling of the cells and delayed cascade of enzyme activation, finally leading to cell death (8).

The immature brain is particularly vulnerable to the effects of glutamate release due to greater density and proportion of the NMDA receptor sites compared to the adult brain (9).

Elevated concentrations of excitotoxic amino acids in the cerebrospinal fluid (CSF) and brain interstitial fluid have also been reported in both experimental (10, 11) and clinical studies of bacterial meningitis (8), and the administration of glutamate receptor antagonist kynurenic acid attenuated the neuronal injury in experimental bacterial meningitis (12). These findings suggest that excitotoxicity may mediate the neuronal injury and that NMDA antagonist may be an attractive new adjuvant therapeutic agent to attenuate the acute inflammatory responses and to ameliorate neuronal injury in neonatal bacterial meningitis.

In the present study, we evaluated the efficacy of noncompetitive NMDA antagonist MK-801 (dizocilpine) as an adjuvant therapy in attenuating the acute inflammatory responses-

es and ameliorating brain damage in neonatal bacterial meningitis. We tested the hypothesis that MK-801 attenuates the acute inflammatory responses and ameliorates the development of brain injury in experimental *Escherichia coli* meningitis in the newborn piglet. In this study, we used the newborn piglet as an animal model of neonatal bacterial meningitis because the piglet brain is comparable to human brain at birth in growth velocity, energy metabolism and vascular anatomy (13, 14). *E. coli* was used to induce meningitis because it is the most common Gram-negative pathogen of neonatal bacterial meningitis (1). Inflammatory responses were assessed by monitoring changes in intracranial pressure (ICP), tumor necrosis factor- α (TNF- α) level, glucose and lactate concentrations, and leukocyte counts in the CSF. Meningitis-induced alterations in brain cell membrane structure, function, and energy stores were determined by measuring lipid peroxidation products (conjugated dienes), Na⁺, K⁺-ATPase activity, and concentrations of high-energy phosphate compounds (ATP and phosphocreatine) in the cerebral cortex, respectively.

MATERIALS AND METHODS

Animal preparation

The experiments described herein were reviewed and approved by the Institutional Animal Care and Use Committee of the Samsung Biomedical Research Center, Seoul, Korea. This study followed the institutional and National Institutes of Health guidelines for laboratory animal care.

Newborn piglets of mixed strain (Yorkshire, conventional breed purchased from Paju farm, Paju, Kyunggi-Do, Korea) less than 3 days were used in this study. Animals were inhaled with ether for sedation and anesthesia was induced with sodium thiopental 5 mg/kg intravenously and supplemental doses were given as required to maintain anesthesia. After local injection with lidocaine (1%), tracheostomy was performed and the piglet was paralyzed with pancuronium 0.1 mg/kg intravenously and ventilated with neonatal pressure limited-time cycled mechanical ventilator (Sechrist Infant Ventilator, IV-100B, Sechrist Industries Co., Anaheim, CA, U.S.A.). Ventilator settings were adjusted to keep arterial PO₂ at 80-100 mmHg, and PCO₂ at 35-45 mmHg. Femoral arteries and veins were cannulated for blood pressure monitoring, blood sampling, and for medication and fluid infusion, respectively. Heart rate, ECG, oxygen saturation, ICP and blood pressure were continuously monitored using Hewlett Packard neonatal monitoring system (Hewlett Packard Model M12-76A, Hewlett Packard Co., MA, U.S.A.). Cisternal puncture was done with a 22 gauge spinal needle (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) and the needle was kept in situ for continuous ICP monitoring and intermittent CSF sampling. Throughout the experiment the piglet was placed

under the servo-controlled warmer (Airshields Inc., Hatboro, PA, U.S.A.) and rectal temperature was maintained between 38.0 and 39.0°C.

Bacterial preparation

E. coli strain used in this study was EC69 strain (gift of Dr. Kwang Sik Kim, University of Southern California, Los Angeles, Calif., U.S.A.). EC69 strain was generated by P1 transduction of the *E. coli* K12 outer membrane protein A (Omp A) gene to Omp A-mutant of RS218, a CSF isolate from a newborn with *E. coli* meningitis (15). The organism was cultured overnight in 10 mL of brain heart infusion broth (BHI, Difco Laboratories, Detroit, Mich., U.S.A.), diluted in fresh medium, and grown for 1 hr to mild-logarithmic phase.

The suspension was centrifuged for 10 min at 5,000 g and resuspended in normal saline, and the absorbance was measured to adjust the bacterial density to the desired concentration.

Experimental protocol

After the surgery and stabilization period, 44 newborn piglets were randomly divided into three groups; 9 in the normal control group (NC), 21 in the meningitis control group (MC), and 14 in the meningitis with MK-801 treatment group (MK). Meningitis was induced by injection of 10⁸ colony forming units (CFU) of *E. coli* in 100 μ L of saline into the cisterna magna. In MK, 3 mg/kg of MK-801 (Sigma, St. Louis, Mo., U.S.A.) in distilled water was given as a bolus intravenous injection 30 min before intracisternal bacterial inoculation. Heart rate, ECG, ICP, blood pressure, and oxygen saturation were continuously monitored during the experiment.

Arterial blood gas analyses, glucose, lactate concentrations in the blood and CSF were measured at baseline and every hour for 4 hr after the bacterial inoculation. Bacterial titers in the CSF were measured by plating 10-fold dilutions on blood agar plates overnight at 37°C in room air. CSF leukocyte counts were measured using hemocytometer, and TNF- α concentrations were measured with commercially available ELISA kit for human TNF- α (Predicta®, Genzyme, Cambridge, Mass., U.S.A.). Arterial blood gases were measured on a blood gas analyzer (Ciba-Corning Diagnostics Corp., Medfield, MA, U.S.A.) and concentrations of glucose and lactate were measured using a YSI model 2300 dual analyzer (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.). CSF concentrations of amino acids at baseline and 4 hr after the induction of meningitis were measured by HPLC (chromatograph HP 1090, Hewlett Packard, Santa Clara, CA).

The CSF samples were deproteinized with sulfosalicylic acid and CSF amino acids were determined with a 451 Alpha Plus amino acid analyzer (LKB Biochrome, Cambridge, U.K.). At the end of the experiment, brain cortex was rapidly harvest-

ed using guillotine, frozen in liquid nitrogen, and stored at 80°C for further biochemical analyses.

Biochemical analyses of brain cortex

Methods of preparing brain cell membrane and determining cerebral cortical cell membrane Na⁺, K⁺-ATPase activity, levels of conjugated dienes, tissue glucose and lactate concentrations, ATP and phosphocreatine (PCr) were described previously in detail (16-18). Briefly, brain cell membranes were prepared according to the method described by Harik *et al.* (19). The activity of cerebral cortical cell membrane Na⁺, K⁺-ATPase was determined by subtracting the enzyme activity in the presence of ouabain from the total activity in the absence of ouabain (20). The level of conjugated dienes was determined by using the method of Recknagel and Glende (21). The concentrations of glucose and lactate in the cerebral cortex were determined spectrophotometrically by using a commercially available kit (Sigma Diagnostics). Brain concentrations of ATP and PCr were determined with a coupled enzyme assay, using the method of Lamprecht *et al.* (22).

Statistical analysis

Data were given as mean \pm standard error (SE). Data were analyzed by unpaired t-test for intergroup comparisons. To detect significant changes over time within each group, data were compared by using repeated measures analysis. Statistical analyses described above were done by using SPSS software program version 10.0. A *p*-value of <0.05 was considered significant.

Table 1. Physiological data at 4 hr of experiment in each group of newborn piglets

	NC (n=9)	MC (n=21)	MK (n=14)
Heart Rate (beats/min)	204 \pm 12	186 \pm 7	179 \pm 9
Arterial base excess (mEq/L)	2.31 \pm 1.04	-1.51 \pm 1.29	-1.38 \pm 0.89
Arterial pH	7.47 \pm 0.05	7.38 \pm 0.02	7.38 \pm 0.02
Blood glucose (mg/dL)	95.8 \pm 19.1	75.7 \pm 6.4	89.3 \pm 5.4
CSF glucose (mg/dL)	64.2 \pm 5.5	32.9 \pm 6.9*	39.6 \pm 4.4*
Blood lactate (mmol/L)	1.3 \pm 0.2	1.5 \pm 0.2	1.4 \pm 0.1
CSF lactate (mmol/L)	2.5 \pm 0.3	6.9 \pm 0.5*	7.5 \pm 0.4*
Bacterial titer (*10 ⁵ cfu/mL)	0	2.9 \pm 0.6*	2.8 \pm 0.4*
Glutamate in CSF (nmol/L)	36.2 \pm 10.2	123.5 \pm 29.2*	93.3 \pm 21.1*

NC, Normal control group; MC, Meningitis control group; MK, meningitis with MK-801 pretreatment group.

Values given represent mean \pm SE. *: *p* < 0.05 compared to NC.

RESULTS

Physiological variables

No significant differences in physiological values, such as heart rate, arterial pH, PaO₂, PaCO₂ and base excess were observed throughout the experiment between the three experimental groups (Table 1).

In NC, there were no significant changes in ICP and mean arterial blood pressure (MABP) (Fig. 1). In MC, ICP increased progressively and became significantly different from corresponding values in NC after 1 hr. Increased ICP in MC was not attenuated in MK. In MC, at 3 hr of meningitis, MABP was decreased but soon recovered to normal value. In MK, MABP was transiently but significantly increased until 1 hr after the MK-801 injection, and after then returned to normal values. Cerebral perfusion pressure (CPP), calculated as MABP minus ICP, was transiently increased in MK, immediately after the MK-801 injection for 1 hr, and significantly decreased after 2 hr in both MC and MK.

Glucose and Lactate concentrations in the blood, brain, and CSF

There were no significant differences in blood and brain glucose and lactate levels between three experimental groups (Table 1, 2). In MC, CSF glucose level significantly decreased after 3 hr, lactate level significantly increased after 1 hr of meningitis, and these abnormalities were not significantly attenuated in MK (Table 2).

Glutamate concentration, bacterial titer, leukocyte counts and TNF- α levels in CSF

Glutamate concentration in the CSF at baseline was not significantly different between the three experimental groups, and the elevated concentration observed in MC compared to

Table 2. Biochemical data in the cerebral cortex of newborn piglets in each experimental group

	NC (n=9)	MC (n=21)	MK (n=14)
Glucose (mmol/kg)	3.84 \pm 0.32	3.61 \pm 0.23	3.76 \pm 0.31
Lactate (mmol/kg)	3.36 \pm 0.37	4.14 \pm 0.49	3.93 \pm 0.18
Na ⁺ , K ⁺ -ATPase (μ molPi/mg protein/hr)	53.60 \pm 0.99	47.34 \pm 0.77	48.83 \pm 0.46*
Conjugated dienes (μ mol/g protein)	0.82 \pm 0.02	1.05 \pm 0.03*	0.94 \pm 0.02* [†]
ATP (mmol/kg)	3.53 \pm 0.26	3.08 \pm 0.18	3.96 \pm 0.08* [†]
Phosphocreatine (mmol/kg)	3.83 \pm 0.28	2.82 \pm 0.21*	3.94 \pm 0.15 [†]

NC, Normal control group; MC, Meningitis control group; MK, meningitis with MK-801 pretreatment group.

Values given represent mean \pm SE. *: *p* < 0.05 compared to NC.

[†]: *p* < 0.05 compared to MC

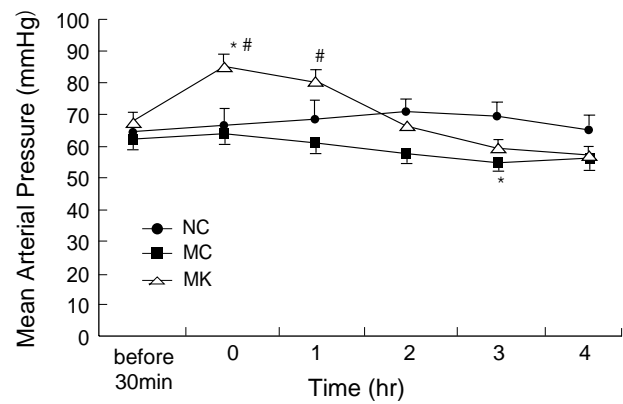
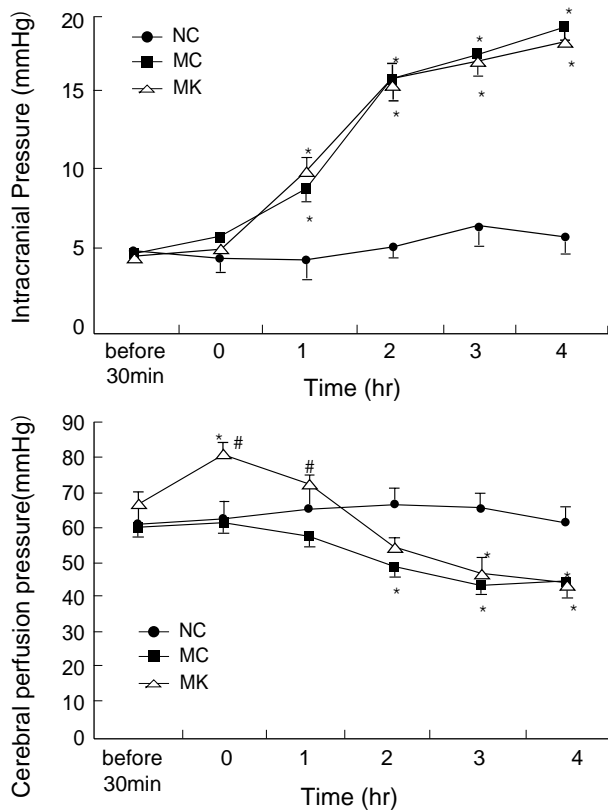


Fig. 1. Time course of intracranial pressure, mean arterial pressure and cerebral perfusion pressure in newborn piglets in each experimental group. NC, Normal control group; MC, Meningitis control group; MK, meningitis with MK-801 pretreatment group. Data are expressed as mean ± SE; * : $p < 0.05$ compared to NC; # : $p < 0.05$ compared to MC.

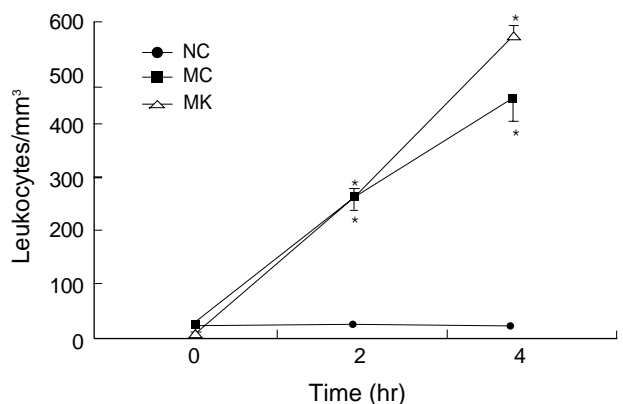
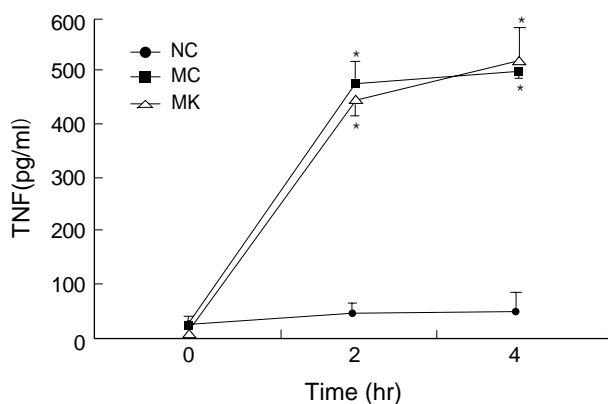


Fig. 2. Changes in tumor necrosis factor- α (TNF- α) and leukocyte counts in the CSF of newborn piglets in each experimental group with time. NC, Normal control group; MC, Meningitis control group; MK, meningitis with MK-801 pretreatment group. Data are expressed as mean ± SE; * : $p < 0.05$ compared to NC; # : $p < 0.05$ compared to MC.

NC at 4 hr after the bacterial inoculation was not significantly attenuated in MK (Table 1). The bacterial titer in the CSF between MC and MK was not significantly different throughout the experiment (Table 1). Leukocyte counts and TNF- α levels in the CSF were significantly elevated at 2 hr and 4 hr in MC, compared to NC ($p=0.000, 0.022$) and these elevations were not significantly attenuated in MK (Fig. 2).

Biochemical data in cerebral cortex

Levels of lipid peroxidation products (conjugated dienes),

measured as an indicator of alterations in cell membrane structure, were significantly elevated in MC compared to NC, and this elevation was significantly attenuated in MK (Table 2). Cerebral cortical cell membrane Na⁺, K⁺-ATPase activity, measured as an index of brain cell membrane function, decreased significantly in MC compared to NC, and this abnormality was not significantly improved in MK. Cerebral ATP levels were not significantly reduced, but PCr was significantly reduced in MC compared to NC ($p=0.015$), and the concentrations of ATP and PCr were significantly increased in MK ($p < 0.01$).

DISCUSSION

In the present study, although it failed to down-modulate the inflammatory parameters such as increased ICP, CSF leukocytosis, increased lactate and TNF- α levels in the CSF, and hypoglycorrhachia observed in the meningitis group, MK-801 pretreatment showed some neuroprotective effects as evidenced by significant attenuation of cerebral lipid peroxidation products (conjugated dienes) and increase of brain high-energy phosphate compounds (ATP and PCr). The failure of MK-801 pretreatment to attenuate the acute inflammation suggest that the role of excitotoxicity in mediating the acute inflammatory responses may not be essential, at least during the early phase of neonatal bacterial meningitis. Leib *et al.* reported the neuroprotective effect of an excitatory amino acid antagonist, kynurenic acid (12). They showed protective effect from excitotoxic neuronal injury through the inhibition of EAA receptors in experimental bacterial meningitis. Reduction of brain edema resulting from EAA inhibition may have more beneficial effects indirectly by reducing intracranial pressure and by improving cerebral blood flow. Neuroprotective effects observed with MK-801 pretreatment in this study and with kynurenic acid in a rat model of group B streptococcal meningitis suggest an important contribution of excitotoxicity to neuronal injury in these animal models of neonatal bacterial meningitis.

Accumulation of cytosolic calcium by the activation of glutamate receptors induces various deleterious events, including especially the generation of oxygen free radicals (23). Oxygen free radicals attack double bonds of polyunsaturated fatty acids in cell membranes in the process called lipid peroxidation, and induce cell injury by altering cell membrane structure and fluidity (24, 25). Therefore, our data of increased lipid peroxidation products (conjugated dienes) observed in MC indicate increased free radical production and the resultant oxidant-induced brain cell membrane injury during bacterial meningitis. Significant attenuation of these abnormalities observed with MK-801 pretreatment in this study support the assumption that oxygen free radicals play a critical role in mediating excitotoxicity-induced neuronal cell death (24, 25).

Oxygen free radicals could deplete cellular energy stores directly by impairing mitochondrial oxidative metabolism (26), or indirectly by activating poly (ADP-ribose) polymerase (27). Therefore, our data of the significant reduction in cerebral high energy phosphate compounds (ATP and PCr) observed in the meningitis group and the significant improvement of these abnormalities with MK-801 pretreatment could be explained by the impairment of mitochondrial oxidative metabolism due to increased production of oxygen free radicals, key mediators of excitotoxicity during bacterial meningitis and significant attenuation of the production of oxygen free radicals with MK-801 pretreatment.

Degradation of cell membrane structure by lipid peroxi-

dation reduces Na⁺, K⁺-ATPase activity and results in brain cell dysfunction that may lead to osmotic cell swelling and neuronal death (28). Therefore, increased levels of lipid peroxidation products (conjugated dienes) might be primarily responsible for the reduced Na⁺, K⁺-ATPase activity observed in MC in this study. Since decreased Na⁺, K⁺-ATPase activity was observed for more than 30% reduction from baseline in high-energy phosphate compounds, reduced Na⁺, K⁺-ATPase activity in MC could not be attributable to cerebral energy depletion. In MK, although increased levels of lipid peroxidation products (conjugated dienes) were significantly attenuated, the improvement in reduced Na⁺, K⁺-ATPase activity failed to reach a statistical significance.

Excitotoxicity is based on the release of EAA, mainly glutamate, and is an important concept to explain the pathophysiology of brain ischemia (4, 5). Hypoxia-ischemia can disrupt the function of excitatory synapses with depletion of energy phosphate, leading to the accumulation of extracellular glutamate, combined with opening of channels operated by glutamate receptors. Excessive synaptic glutamate concentrations result from both the excessive presynaptic release and the failure of reuptake, due to the energy failure of ion exchange systems and resultant membrane depolarization (29, 30). In bacterial meningitis, inflammation in subarachnoid space surrounds blood vessels, and the resulting vasculitis causes temporary vasospasm (31-33). Brain edema, increased ICP, systemic hypotension, and impaired cerebral blood flow autoregulation can lead to a decrease in cerebral blood flow (34). These changes result in local ischemic damage with subsequent glutamate release and development of excitotoxic injury like hypoxic ischemic encephalopathy. However, our data of significant increase in glutamate concentration without systemic hypotension in MC, and the failure of significant attenuation of these abnormalities despite significant improvement in cerebral energy stores in MK suggest that excitotoxicity can occur without global ischemia and widespread depletion in cerebral energy stores during the early phase of bacterial meningitis. Energy depletion at the cellular level due to acute inflammatory responses and anaerobic glycolysis might be responsible for these abnormalities. Further studies will be necessary to clarify this. Transient but significant increase in MABP observed with MK-801 pretreatment could be explained by its sympathetic nervous system stimulatory effects (35). However, whether this effect is related to some neuroprotective effects of MK-801 is not clear.

In summary, our results suggest that MK-801 pretreatment may have some neuroprotective effects in experimental neonatal bacterial meningitis although it failed to attenuate the acute inflammatory responses.

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